EXHIBIT A

Novartis Pharma Research

Construction and Characterization of A Recombinant Adenoviral Vector Encoding Secreted Murine Endostatin *in vitro*

Therapeutic Area: GTI, Tumor Targeting Program

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Document type: Internal study Report

Document status: Draft

Release date:

Number of pages: 19

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Abbreviations

Abbreviation	Description
Sig-mEndo	Ig-k leader and murine endostatin chimeric
mEndo	murine endostatin containing supernatant protein from Av3mEndo transduced cells
NullI	control supernatant protein from Av3NullI transduced cells
Av3	third generation, E1, E2a, and E3 deficient adenoviral vector
SV4	simian virus 40
RCA	replication competent adenovirus
FBS	fetal bovine serum
pfu	plaque forming units
HBSS	Hanks' balanced salt solution
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Summary

The replication deficient recombinant adenoviral vector, Av3mEndo, encoding murine endostatin in a secreted form was prepared in an Av3 backbone gene with E1, E2a, and E3 deletion. Genomic DNA characterization confirmed the correct construction of Av3mEndo. The expression and secretion of murine endostatin was demonstrated in Av3mEndo transduced Hep3B (human hepatoma), and S8 (human lung carcinoma derived) cells. N-terminal protein sequencing analysis demonstrated that murine endostain was cleaved from signal peptide at the expected site with 80% containing Asp-Ala-Ala, and 20% containing Ala from the signal peptide. The Heparin-bound supernatant protein of Av3mEndo transduced cells demonstrated a complete inhibition of VEGF165 induced HUVEC migration at 10 ng/ml, while the control treated with Av3NullI vector showed no effect. Results supported that the constructed Av3mEndo secreted biologically active endostatin.

Introduction

Angiogensis, the process of new blood vessels formation from the existing vessels, is known to be important for tumor growth (Folkman, 1971). Based on this, the anti-angiogenesis have been extensively studied in cancer therapy application over the decade. Numerous anti-angiogenesis approaches have been reported and reviewed by Zetter (1998). These include (1) naturally occurring angiogenic inhibitors, e.g. Thrombospondin, α-Interferon, plalate factor VI, metalloproteinase inhibitor; (2) synthetic angiogenesis inhibitors, e.g. synthetic protease inhibitors, anti-adhesive peptides (cRGD peptide), anti-integrin antibody (directed against αvβ3); (3) pharmacological inhibitor agents, e.g. TNP470, Thalidomide, Carboxyamidotrizaole (CAI); and (4) tumor-derived inhibitors, e.g. angiostatin, endostatin. Others are currently either in clinical trial or under development, e.g. anti-signaling agents, ribozyme inhibiting VEGF receptor synthesis.

Among them, endostatin has been one of the inhibitors demonstrated the most dramatic anti-tumor effect through systemic protein administration (O'Reilly, *et al.* 1997). After several cycles of treatment, the tumors were at their dormancy ultimate with complete tumor arrest. No drug resistance or side effect was reported (O'Reilly, *et al.* 1997). However, like most angiogenic inhibitors, it functions through cytostatic rather cytotoxic effect relying on a prolong maintenance of anti-angiogenesis state. Direct protein injection may not be sufficient for prolong maintenance of anti-angiogenesis state and also likely to be too costly and cumbersome. Thus, the gene therapy approach for anti-angiogenic protein delivery was thought to be more applicable and realistic.

Endostatin is a internal carboxy terminal peptide of collagen XVIII (O'Reilly, *et al.* 1997). The crystal structure has been well characterized (Hohenester, *et al.*, 1998). The protein has been demonstrated inhibition of endothelial cell proliferation *in vitro* and potent antitumor effect *in vivo* in several independent groups (O'Reilly, *et al.* 1997, Mohanraj, *et al*, 1999, Wang, et al., 1999). Currently, Phase I clinical trial with endostatin protein administration by EntreMed is in planning. The molecular mechanism of endostatin induced anti-angiogenesis is not clear. Although antiangiogenic gene therapy approach has been reported in several studies with various angiogenic inhibitors, e.g. angiostatin (Toshihide, *et al.*, 1998, Griscelli, et al., 1998, Nguyen, *et al.*, 1998), plalate factor 4 (Toshihide, *et al.*, 1997), endostatin (Nguyen, *et al.*, 1998), antisense mRNA against VEGF (Nguyen, *et al.*, 1998), and soluble flt-1 (Kong, *et al.*, 1998), most of them were designed for in situ delivery. Recent report by Wang, *et al.* (1999) showed that muscle injection of plasmid encoding endostain could produce circulating endostatin. However, the highest level reached was around 8 ng/ml which might fall short for anti-tumor therapy. GTI has demonstrated sustained level of Factor VIII expression in Hemophilia project using Av3 vector through liver transduction. Systemic delivery of Av3 vector encoding angiogenic inhibitor might provide sustained therapeutic level of expression to prolong anti-angiogenesis state. The current study is designed as a proof of concept study (1) to construct the Av3 vector encoding endostain, and (2) to characterize its biological function *in vitro*.

Materials and Methods

Cell lines

Human umbilical vein endothelial cells (HUVEC) were from Cascade Biologics, Inc. and were cultured in M200 supplemented with Growth Supplement for Large Vessel Endothelial Cells (LSGS) (Cascade Biologics, Inc). A549 (human lung carcinoma), Hep3B (human hepatocellular carcinoma), 293 ((human embryonic kidney) cells were obtained from ATCC and routinely cultured in Richter's CM with 5% FBS; EMEM with 10% FBS; and Richter's CM with 10% FBS respectively. S8 (derived from A549 cells) were made at GTI and routinely cultured in Richter's CM with 5% FBS.

PCR and assembly of murine endostatin cDNA and Ig-κ leader sequence

The mouse endostatin cDNA was PCR amplified from mouse collagen XVIII clone ID #748987 from GenomeSystems, Inc. with the primers of 5'-ACT GGT GAC GCG GCC CAT ACT CAT CAG GAC TTT CAG CC-3' and 5'- AAG GGC TAT CGA TCT AGC TGG CAG AGG CCT AT-3' (598 bp F1 fragment). The mouse Ig-κ leader was PCR amplified from pSecTag2 (Invitrogen) with the primers of 5'- CAC TGC TTA CTG GCT TAT CG-3' and 5'- CTG ATG AGT ATG GGC CGC GTC ACC AGT GG-3' (147 bp F2 fragment). PCR was carried out with Pfu DNA polymerase (Stratagene) for 35 cycles in the following conditions: 95°C hot start for 3 min, 95°C denaturation for 1 min, 55°C annealing for 1 min, and 72°C extension for 2 min. The DNA fragments were gel purified.

The sig-mEndo chimeric DNA (718 bp) was generated by PCR splice overlap extension (Horton, et al., 1990) with F1 and F2 DNA fragments generated above as templates to assemble mouse Ig-κ leader sequence and murine endostatin cDNA. PCR was carried out with the primers of 5'-ACT GGT GAC GCG GCC CAT ACT CAT CAG GAC TTT CAG CC-3' and 5'- CTG ATG AGT ATG GGC CGC GTC ACC AGT GG-3' using Pfu DNA polymerase (Stratagene). PCR was run for 35 cycles in the following conditions: 95°C hot start for 3 min, 95°C denaturation for 1 min, 60°C annealing for 1 min, and 72°C extension for 2 min.

The 718 bp sig-mEndo chimeric DNA fragment was gel purified and cloned into the Nhe1 and ClaI sites of the adenoviral shuttle plasmid, pAvF9lxr, to create pAvmEndoLxr. The sig-mEndo chimeric DNA was cloned downstream of the RSV promoter and the adenoviral tripartite leader and included the SV40 polyadenylation signal, a homologous recombination region, and Loxp site for Cre/Lox mediate recombination. The entire region of the sig-mEndo in the pAvmEndoLxr plasmid was confirmed with the direct sequencing analysis by Gene Therapy Core Technologies Molecular Core Laboratory.

Construction of recombinant adenoviral vectors, Av3mEndo

The recombinant Av3mEndo (with E1, E2a, and E3-delection) encoding the sig-mEndo chimeric was generated by "Quick Cre/Lox two plasmid system" in the following procedure. The pAvmEndoLxr and pSQ3 were first linearlized with NotI and ClaI restriction enzymes,

respectively. The transient transfection was performed on the 6-well plate at 4 x 10^5 of 293 cells per well using the calcium phosphate mammalian transfection system (Promega Corporation, Madison, WI). The calcium phosphate-DNA precipitate was prepared with 4.8 μg of linearlized pAvmEndoLxr, 12 μg of linearized pSQ3, 6 μg of pcmvCre, and 6 μg of pcmvE2a in a total volume of 1.8 ml. A 0.6 ml of calcium phosphate-DNA precipitate was added to each well. The 293 cells were incubated with calcium phosphate-DNA precipitate at 37°C for 16 hours. The precipitate was removed and the cells were washed with PBS. Fifteen days post transfection, the cytopathic effect (CPE) was observed. The cells and the medium were then harvested by scrapping. The crude viron lysate was prepared by five cycles of freezing and thawing. The Av3mEndo vector was re-amplified in S8 cells with 0.3 μM Dexamethazone in Richcter's CM medium containing 5% FBS until CPE was observed.

Av3mEndo research seed lot characterization

The research seedlot purified Av3mEndo vector was scaled up by GTI Gene Therapy Core Technologies. The adenoviral vector titer (particles/ml) and biological titer (pfu/ml) were determined as described (Mittereder, *et al.*, 1996) by GTI Gene Therapy Core Technologies. The ratio of total particles to infectious particles (particles/pfu) was calculated. The purity of research seedlot preparation, defined as an assessment of possible contamination with replication competent adenovirus (RCA) was assessed by GTI Gene Therapy Core Technologies. The Av3mEndo seedlot was determined to be negative for RCA.

Characterization of recombinant Av3mEndo

Various restriction digests by XmnI, HindIII, ClaI, and BamHI of the purified viral DNA of Av3mEndo, and Av3NullI were compared on 1.0% agarose-TAE gel to verify the genome structure and purity. Southern bolt analysis from the duplicate run was carried out following the standard protocol. After transferred to a nylon membrane, the membrane was prehybridized in 0.5 M $NaPO_4$, 1 mM EDTA, 0.5% BSA, 7% SDS at 65°C for 2 hours. The sig-mEndo internal probe for both Southern and Northern analyses was prepared from pAvmEndoLxr digested by XbaI and StuI at 37°C for 4 hours and gel purified. The membrane was then hybridized with a 554 bp [^{32}p]-labeled sig-mEndo internal probe at 65°C and washed in SSC/SDS containing buffers at 65°C following the standard protocol. Membrane was exposed to film for one hour at room temperature.

Northern blot analysis

A549 cells were transduced with Av3mEndo, or control Av3NullI vector at the pfu to cell ratio of 1. Forty-eight hours post transduction, the cell pellets were harvested and total RNA was isolated using the RNAzol B (Tel-Test) extraction method. Northern blot analysis was carried out according the standard procedure. A 20 μg aliquot of total RNA was analyzed on 1% Agarose formaldehyde/MOPS gel. RNA was transferred to a nylon membrane and prehydridized in Zip Hyb Solution at 60°C for 45 min. The membrane was then hybridized with a 554 bp [^{32}p]-labeled sig-mEndo internal probe at 65°C and washed in SSC/SDS containing buffers at 65 °C following the standard protocol. Membrane was exposed to x-ray film for 18 hours at -70°C.

Preparation of mEndo and NulI supernatant proteins from vector trausduced cells

The mEndo and NulI supernatant proteins were prepared from Av3mEndo and Av3NulI transduced S8 or Hep3B cells, respectively. Seventy-two hours post transduction, the supernatant was collected and filtered through 2 μ filter. Each 40 ml of supernatant was passed through 1-ml Heparine Sepharose CL-6B (Pharmacia) equilibrated with 50 mM Tris-Cl, pH 7.5, 0.1 M NaCl, and 10% glycerol. After unbound protein washed with 50 mM Tris-Cl, pH 7.5, 0.1 M NaCl, and 10% glycerol, the Heparine column bound protein was eluted with 4-ml buffer containing 50 mM Tris-Cl, pH 7.5, 1 M NaCl, and 20% glycerol. The protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad). The supernatant protein was aliquoted and stored at -70°C until used. The protein was routinely dialyzed against HBSS before used for migration assay analysis.

SDS-PAGE and N-terminal protein sequencing analyses

The prepared mEndo and NulI supernatant protein were analyzed by SDS-PAGE. Each 60-μg of supernatant protein was mixed with Laemmli sample buffer (Sigma) and heated at 95°C for 3 min. The denatured protein was loaded on a 4 -12% linear gradient pre-casted gel (Bio-Rad). The gel was stained with Gelcode blue stain reagent (VWR) to visualize the protein bands. For mEndo N-terminal protein sequencing analysis, the protein was analyzed on SDS-PAGE in a similar way except that the protein was transferred to PVDF membrane (Bio-Rad). The membrane was stained with 0.1% Commassie blue R-250 in 40% methanol and 1% acetic acid for 2 min followed by 4 destaining washes with 50% methanol for 15 min per wash. The membrane was air-dried and the 20 Kd protein band corresponding to murine endostatin was subjected to N-terminal protein sequencing analysis by automated Edman degradation using Perkin-Elmer Applied Biosystems (Protein Sequencing Midwest Analytical, Inc.).

ELISA detection of mEndo secretion

Secretion of murine endostatin was routinely determined by murine endostatin ELISA kit (CytImmune Sciences, Inc., Collage Park, MD) according to the manufacture procedure. Following the plate washed, the analysis was carried out in triplicate on 96-well ELISA plate with the unknown samples diluted by 4, 16, or 64-fold with final 50% of diluent 1 and 25% of diluent 2. Each 100-μl of mEndo standard and diluted unknown sample was dispensed into designated well. Following addition of biotin mEndo-conjugate and anti-mEndo antibody, the plates were incubated at room temperature for 3 hours. After the plate washed, the streptavidin-alkaline phosphatase was added. The plate was incubated at room temperature for 30 min followed by 20-min color development in the streptavidin-alkaline phosphatase reaction mixture. The absorbance was determined at 492 nm by ELISA reader (Bio-Rad). The standard curve was established and the concentration of the unknown samples was determined by extrapolation from murine endostatin standard curve.

Migration assay

Cell migration was assessed in 48-well chemotaxi chambers (Neuroprobe, Cabin John, MD) as described (Polverine, et al., 1991). Polycarbonate (8 μ) membrane (Costar) was coated with bovine collagen type I in the following procedure. Polycarbonate membrane was soaked in 0.5 M acetic acid overnight. The membrane was rinsed with PBS. Collagen type I (bovine) was diluted in 0.2 N acetic acid to the final concentration of 0.1 mg/ml. The membrane was soaked in 0.1 mg/ml collagen for 2 min and air-dried. Low passage (passage 1 to 4) of HUVEC were cultured in M200 supplemented with LSGS (Cascade) until migration assay. Cells were suspended in migration assay medium, M199 plus 1% FBS, to the cell density of 2×10^5 cells/ml and pre-incubated in the presence or absence of tested supernatant proteins, mEndo or NulI in 37°C 5% CO_2 incubator for 30 min. VEGF165 (R&D systems) at various concentrations was prepared in migration assay medium and added into the bottom chamber. After assembly with collagen type I coated polycarbonate membrane in between top and bottom chamber, a 50 μl of pre-incubated cell suspension was added to the top chamber. The membrane was removed after 5 hours of incubation and was stained with Diff-Quik (VWR). The seeded cells on the top chamber was removed by wiping with tissue. Cells that migrated through the membrane to the bottom chamber were counted using BioQuan immage system under the microscope. The basal migration was determined with migration assay medium in the absence of any tested substance, mEndo, NulI, or angiogenic factor, VEGF165.

Results and Discussion

Generation of a recombinant adenoviral vector encoding murine endostatin

Murine endostatin cDNA was PCR generated from the C-terminus of mouse α1(XVIII) collagen clone ID #748987 from GenomeSystems. The cDNA was assembled with murine Ig-κ leader to generate sig-mEndo chimeric for the secretion of murine endostatin protein by PCR splice overlap extension (Horton, et al., 1990). The sig-mEndo chimeric DNA was cloned into the Nhe1 and ClaI sites of the adenoviral shuttle plasmid, pAvF9lxr to create pAvmEndoLxr (Fig. 1A). The entire sig-mEndo chimeric sequence was confirmed by auto sequencing analysis. The consensus sequence and derived protein sequence are shown in Fig 1B. The adenoviral vector encoding sig-mEndo chimeric was generated by "Quick Cre/Lox 2 plasmid system" in 293 cells by transient transfection with pcmvE2a, pCre, pSQ3 and pAv3mEndoLx through Cre/Lox mediated recombination. The generated vector was RCA negative. The correct genome structure of generated Av3mEndo vector was confirmed by restriction digests and Southern blot analysis (Fig 2 A and B).

Expression and secretion of murine endostatin

Av3mEndo mediated murine endostatin expression was characterized by Northern blot analysis in vector transduced A549 cells (Fig 4). The expected sig-mEndo message was only identified in Av3mEndo but not in control, untreated or Av3NulI vector transduced cells. The expression and secretion of murine endostatin was further characterized in vector transduced S8 cells. As shown in Fig. 5, the supernatant proteins of Av3mEndo and Av3NulI vector

transduced S8 cells were compared on SDS-PAGE. A distinct 20 Kd protein band corresponding to the correct size of murine endostatin was only identified in Av3mEndo but not in Av3NullI transduced cells. This 20 Kd protein was subjected to N-terminal protein sequencing analysis and demonstrated the cleavage of the signal peptide from murine endostatin at the expected site. The secretion of murine endostatin was also characterized in Av3mEndo transduced Hep3B cells and demonstrated the endostatin expression and secretion (Fig. 6).

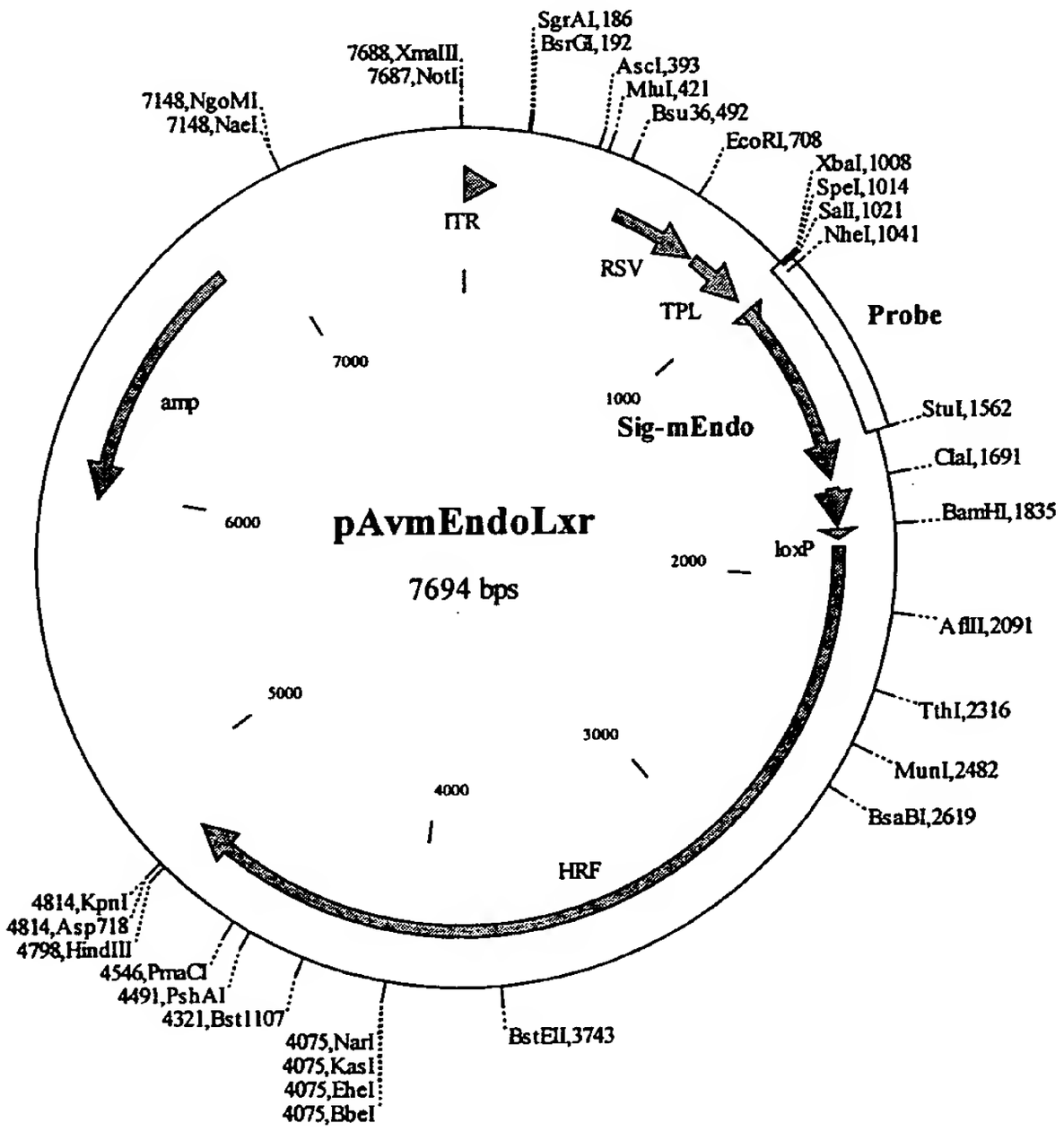
Functional characterization of murine endostatin *in vitro*

The biological activity of the mEndo supernatant protein was characterized in both HUVEC proliferation and migration assay. No inhibition was observed in bFGF induced HUVEC proliferation assay in the presence of mEndo supernatant protein (data not shown). However, mEndo supernatant protein demonstrated potent inhibition effect against VEGF165 induced HUVEC migration in comparison with no effect by NullI supernatant protein as shown in Fig. 7.

Figure 1: Sequence of the murine endostatin and protein.

The mouse Ig-κ leader sequence (sig) was constructed immediate upstream of 5'-of murine endostatin (mEndo) cDNA with linker encoding two analine residues in between for cleavage. The sig-mEndo chimeric DNA, containing leader sequence and murine endostatin cDNA was cloned into the Nhe1 and ClaI sites of the adenoviral shuttle plasmid, pAvF9lxr to create pAvmEndoLxr (Panel A). The sig-mEndo DNA was cloned downstream of the RSV promoter and the adenoviral tripartite leader and included the SV40 polyadenylation signal, a homologous recombination region, and LoxP site for Cre/Lox mediate recombination. The plasmid, pAvmEndoLxr was submitted to the Gene Therapy Core Technologies Molecular Core Laboratory for direct sequencing of the sig-mEndo region. The open bar (probe) marked sig-mEndo internal probe used for Southern and Northern analyses. The sig-mEndo consensus sequence and derived amino acid sequence are displayed in Panel B. The sig-mEndo is 624 bps and produces a secreted endostatin with 184 amino acid residues of approximately 20 kilodalton molecular weight.

Panel A:



Panel B:

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ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGG 42
 M  E  T  D  T  L  L  L  W  V  L  L  L  W

GTTCCAGGTTCCACTGGTGACGCGGCCCATACTCATCAGGAC 84
 V  P  G  S  T  G  D  A  A  H  T  H  Q  D

TTTCAGCCAGTGCTCCACCTGGTGGCACTGAACACCCCCCTG 126
 F  Q  P  V  L  H  L  V  A  L  N  T  P  L

TCTGGAGGCATGCGTGGTATCCGTGGAGCAGATTTCCAGTGC 168
 S  G  G  M  R  G  I  R  G  A  D  F  Q  C

TTCCAGCAAGCCCGAGCCGTGGGGCTGTCGGGCACCTTCCGG 210
 F  Q  Q  A  R  A  V  G  L  S  G  T  F  R

GCTTTCCTGTCCTCTAGGCTGCAGGATCTCTATAGCATCGTG 252
 A  F  L  S  S  R  L  Q  D  L  Y  S  I  V

CGCCGTGCTGACCGGGGGTCTGTGCCCATCGTCAACCTGAAG 294
 R  R  A  D  R  G  S  V  P  I  V  N  L  K

GACGAGGTGCTATCTCCCAGCTGGGACTCCCTGTTTTCTGGC 336
 D  E  V  L  S  P  S  W  D  S  L  F  S  G

TCCCAGGGTCAAGTGCAACCCGGGGCCCGCATCTTTTCTTTT 378
 S  Q  G  Q  V  Q  P  G  A  R  I  F  S  F

GACGGCAGAGATGTCCTGAGACACCCAGCCTGGCCGCAGAAG 420
 D  G  R  D  V  L  R  H  P  A  W  P  Q  K

AGCGTATGGCACGGCTCGGACCCCAGTGGGCGGAGGCTGATG 462
 S  V  W  H  G  S  D  P  S  G  R  R  L  M

GAGAGTTACTGTGAGACATGGCGAACTGAAACTACTGGGGCT 504
 E  S  Y  C  E  T  W  R  T  E  T  T  G  A

ACAGGTCAGGCCTCCTCCCTGCTGTCAGGCAGGCTCCTGGAA 546
 T  G  Q  A  S  S  L  L  S  G  R  L  L  E

CAGAAAGCTGCGAGCTGCCACAACAGCTACATCGTCCTGTGC 588
 Q  K  A  A  S  C  H  N  S  Y  I  V  L  C

ATTGAGAATAGCTTCATGACCTCTTTCTCCAAATAG 624
 I  E  N  S  F  M  T  S  F  S  K  .
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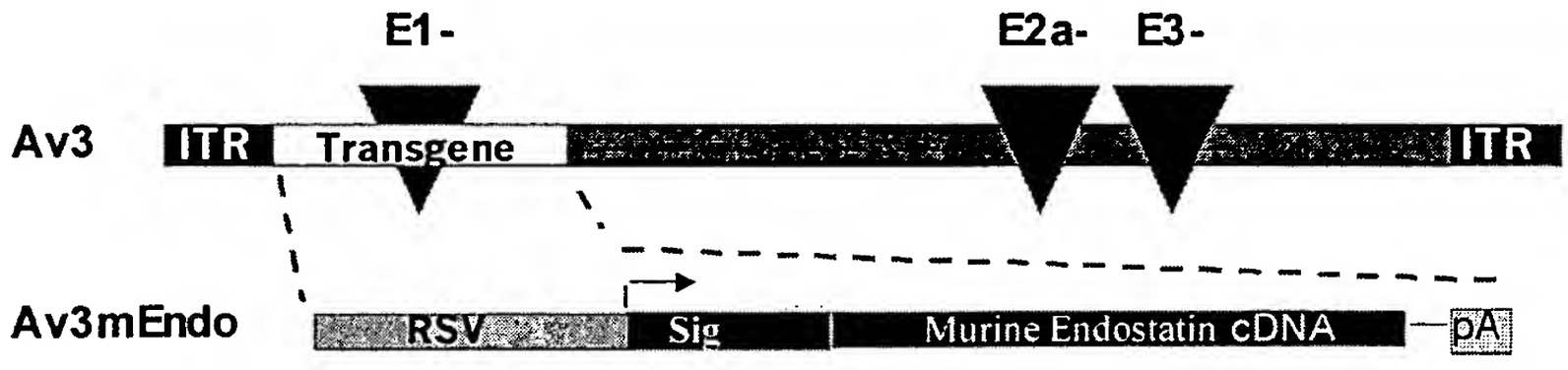
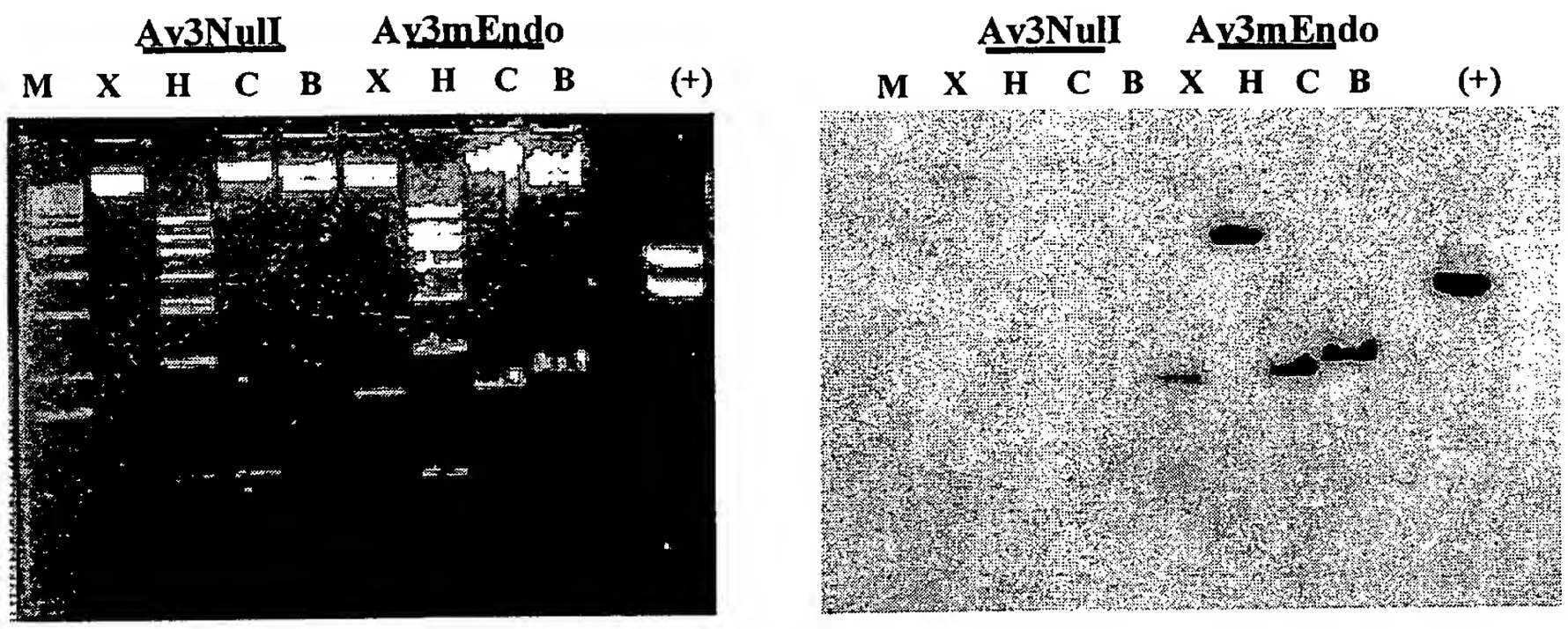
Figure 2: Schematic representation of Av3mEndo, a recombinant adenoviral vector encoding the murine endostaitn cDNA in a secreted form.



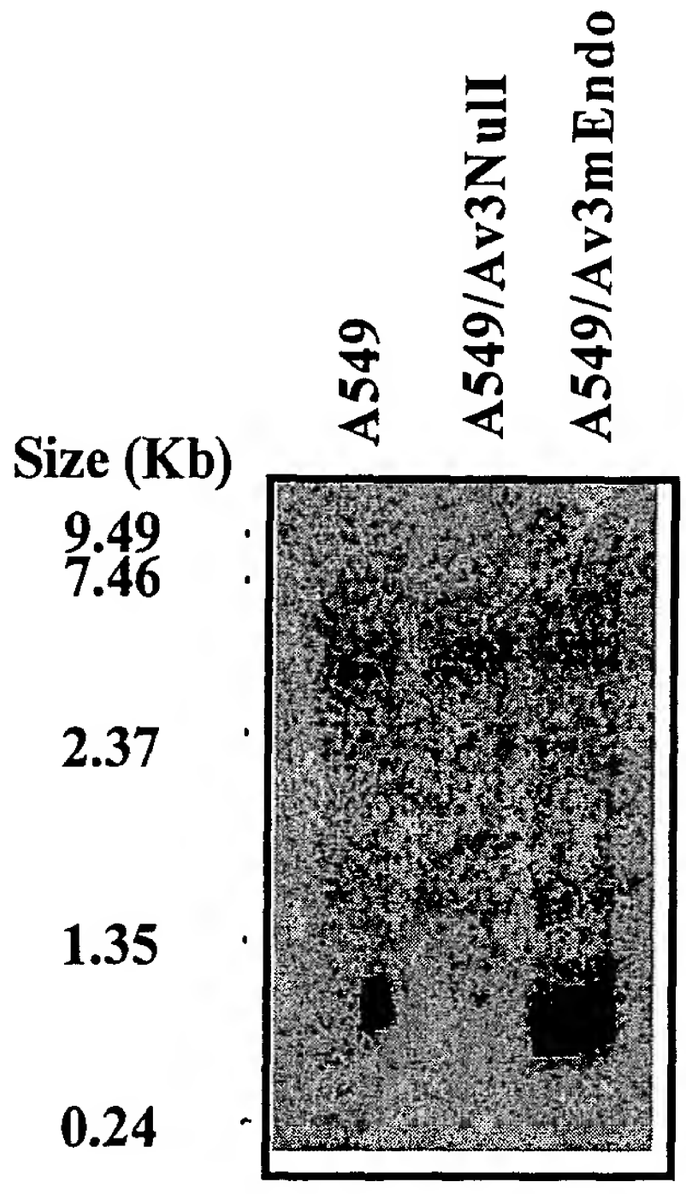
The DNA fragment of murine endostatin cDNA assembled with murine Ig-κ leader (sig-mEndo, 718 bp) was placed downstream of the RSV promoter and the adenoviral tripartite leader. The pAvmEndoLxr was generated with the substitution of F9 cDNA region of the shuttle plasmid pAvF9Lxr by sig-mEndo. The Av3mEndo vector was generated with plasmids of pSQ3 and pAvmEndoLxr by "Quick Cre/Lox 2 plasmid system" with E1, E2a, and E3 deletion. The control vector, Av3NullI, is with the same backbone gene except no sig-mEndo transgene in the expression cassette as shown.

Figure 3: Genomic Characterization of Av3mEndo



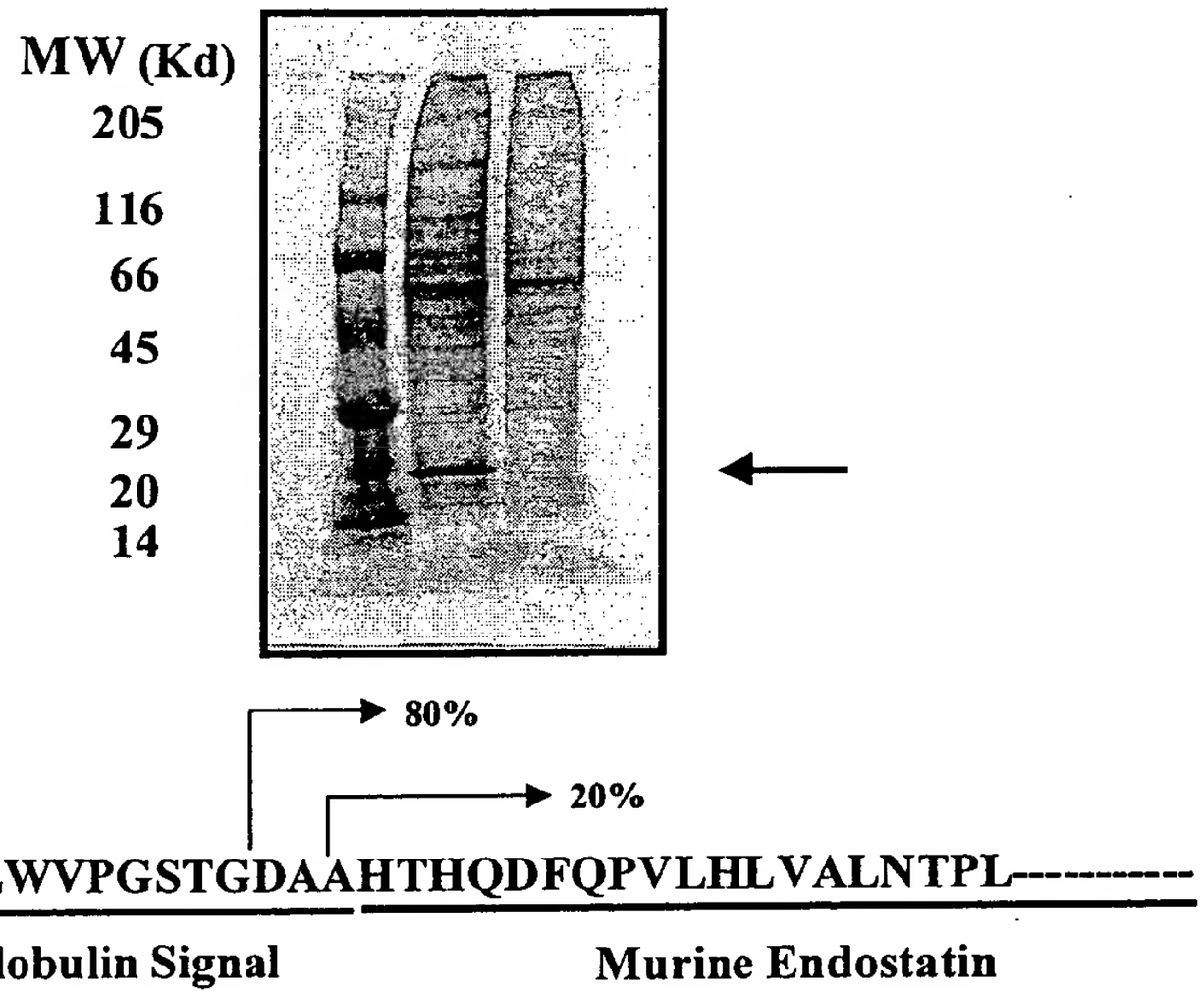
Genomic DNA analysis of Av3mEndo and the control, Av3NullI recombinant vectors was done as described in the text. The isolated DNA was digested with (X) XmnI, (H) HindIII, (C) ClaI, and (B) BamHI. (Panel A) The digested DNA samples (1 μg) were applied to a 1.0% agarose-TAE gel and stained with ethidium bromide to visualize the individual DNA fragments. (M) The 1-kb ladder marker was run in parallel. The pAvmEndoLxr shuttle plasmid encoding murine endostatin was digested with XhoI and used as a positive control (+). (Panel B): The digested DNA fragments on 1.0% agarose-TAE gel shown in Panel A was transferred to a nylon membrane and hybridized with the [^{32}P] labeled 554 bp internal sig-mEndo probe at approximately 180 μci/ml and exposed to film for 1 hour. Southern blot analysis with 554 bp sig-mEndo probe demonstrated the expected hybridization pattern for Av3mEndo.

Figure 4: Adenoviral-mediated expression of murine endostatin in A549 cells: Northern blot analysis.



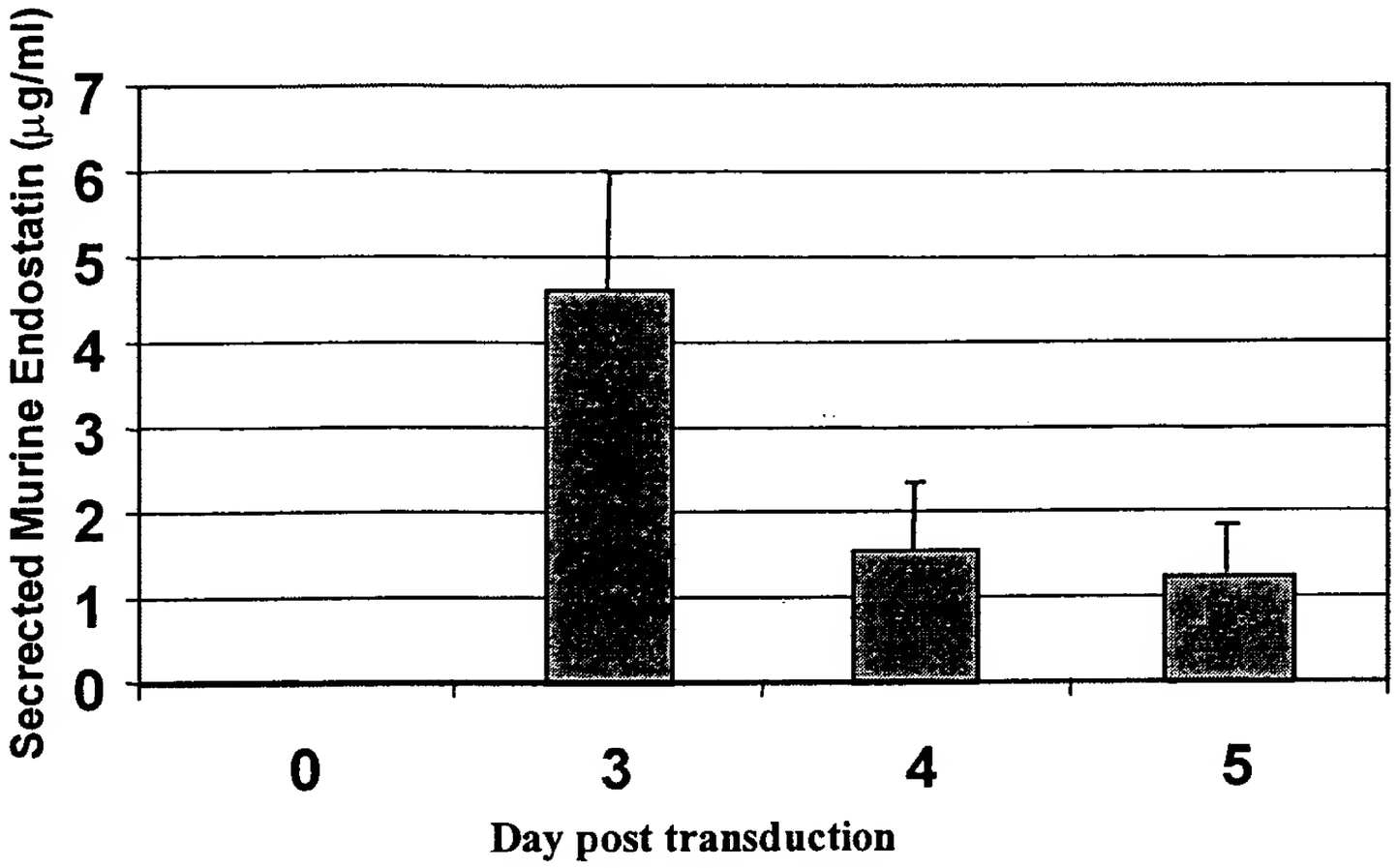
A549 cells were transduced with Av3mEndo or the control, Av3NulI recombinant vectors for 3 hours. Forty-eight hours post transduction, cell pellets were harvested. RNA was isolated and transferred into nylon membrane. The RNA was hybridized with the [^{32}P] labeled 554-bp sig-mEndo internal probe at approximately 6 x 10^6 cpm/ml and exposed to film for 18 hour. Northern blot analysis with 554 bp sig-mEndo internal probe demonstrated murine endostatin (0.8 Kb) was only expressed in Av3mEndo transduced A549 cells but not in untransduced A549 or control vector, Av3NulI transduced A549 cells.

Figure 5: Adenoviral-mediated expression and secretion of murine endostatin in S8 cells: SDS-PAGE analysis of supernatant of transduced cells shows secretion of murine endostatin; N-terminus peptide analysis shows the expected sequence of murine endostaitn.



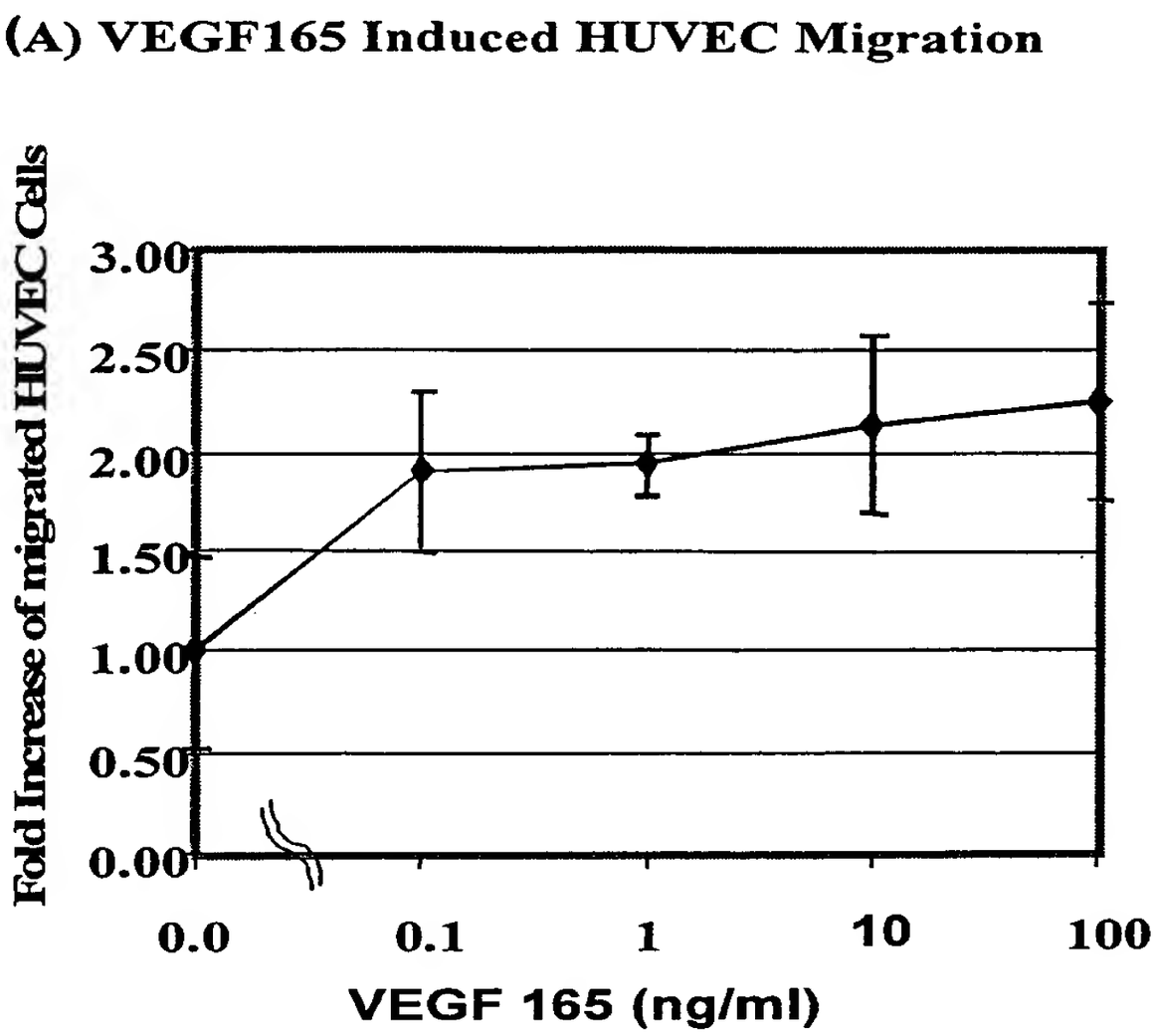
The mEndo (lane 2) and NullI (lane 3) supernatant protein prepared as described in the text were analyzed on SDS-PAGE. Each 60 μg supernatant protein was analyzed on 4 to 12% linear gradient pre-casted gel. The protein standard marker was run on lane 1 as indicated. The gel was stained with Gelcode Blue stain reagent to visualize the protein bands. As indicated, the expected murine endostatin protein band around 20 Kd (marked by arrow) was only generated from Av3mEndo vector but not from the control, Av3NullI vector. After transferred to PVDF membrane from a duplicate SDS-PAGE, the 20 Kd protein band was excised from immoblin membrane blot and subjected to N-terminal protein sequencing analysis. The determined protein sequence result is shown in the bottom with arrows marked as the beginning of the N-terminus of two major secreted proteins, 80% containing additional amino acid residues of DAA, and 20% containing residue of A from murine Ig-κ signal peptide. Results demonstrated that the Av3mEndo transduced S8 cells expressed and secreted murine endostatin after processed from murine Ig-κ signal peptide.

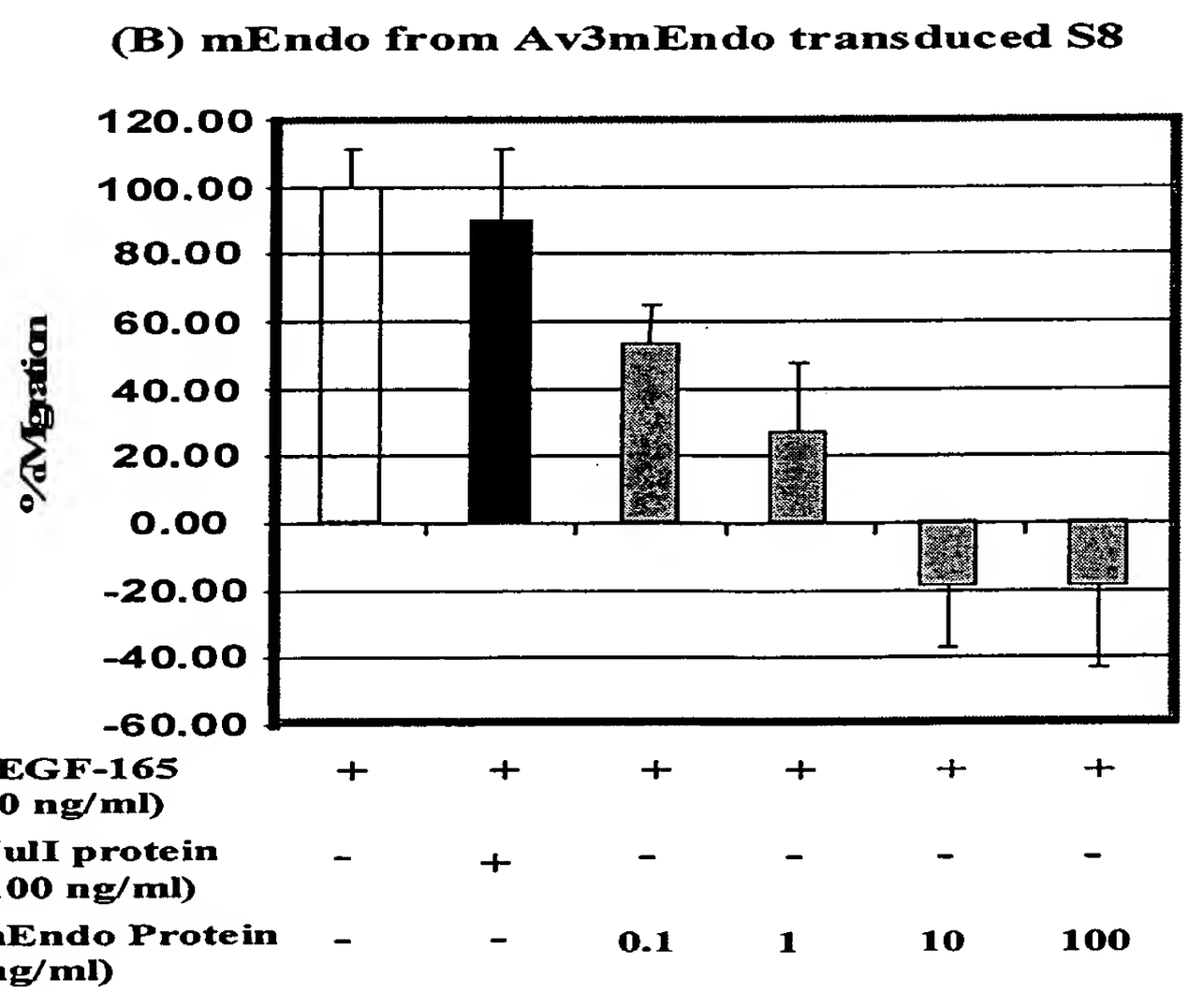
Figure 6: Secretion of murine endostatin from Av3mEndo transduced Hep3B cells.



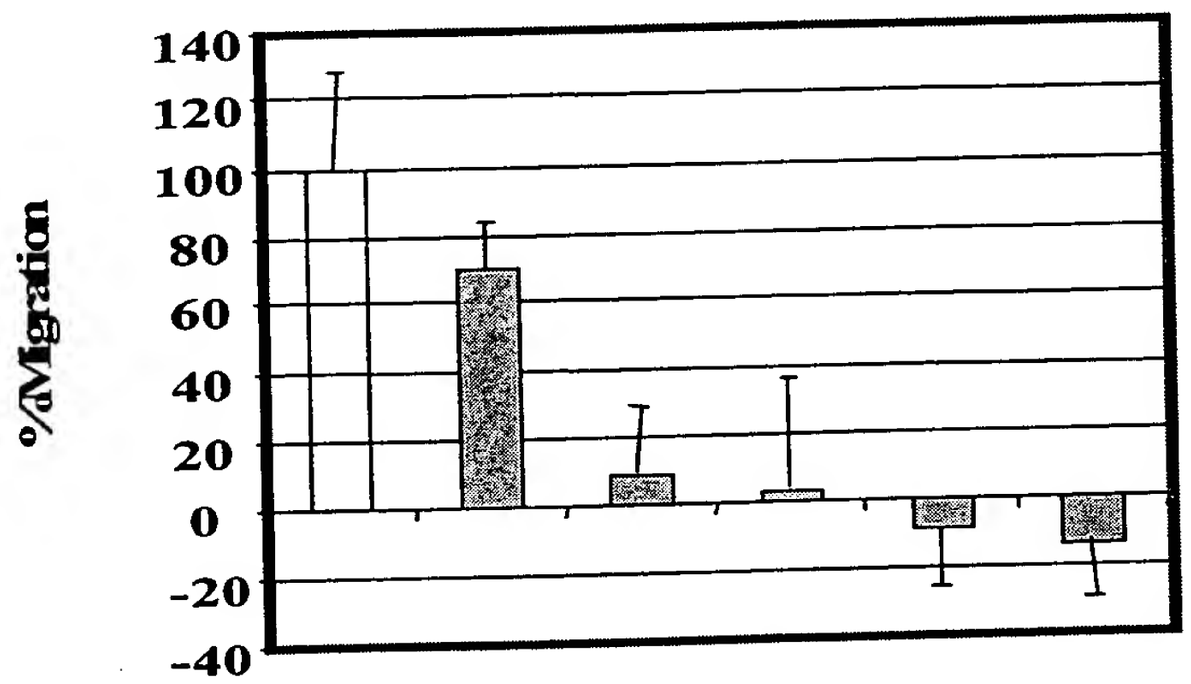
Hep3B cells (10^6) were transduced with Av3mEndo at the pfu to cell ratio of 10 for 3 hours. Followed by replenishment with fresh medium. Supernatant was collected at day 3 post transduction and replenished with fresh medium. Supernatant was then collected at day 4 post transduction and replenished with fresh medium followed by collection at day 5 post transduction. All collected supernatant was subjected to murine endostatin ELISA analysis and demonstrated secretion of murine endostatin from Av3mEndo transduced Hep3B cells.

Figure 7: Functional analysis: inhibition of HUVEC migration by the supernatant protein of the Av3mEndo vector transduced S8 and Hep3B cells.





(C) mEndo from Av3mEndo transduced Hep3B



VEGF-165 (10ng/ml)	+	+	+	+	+	+
mEndo (ng/ml)	-	**0.1**	**1**	**10**	**100**	**1000**

HUVEC migration assay was carried out as described in text. (A) VEGF165 at various concentrations was added to induced HUVEC migration. The migrated HUVEC cells were determined after 5 hours incubation. Fold of increase of migrated HUVEC was calculated relative to the migrated HUVEC in the absence of VEGF165. (B) Supernatant protein mEndo and NullI was prepared from vector transduced S8 cells. (C) Supernatant protein mEndo and NullI was prepared from vector transduced Hep3B cells. Effect of mEndo and NullI supernatant protein to VEGF165 induced HUVEC migration was determined as described in the text. %Migration was calculated based on 100% migration in the presence of 10 ng/ml VEGF165 and in the absence of supernatant protein.

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